



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>5</sup> :</b> <b>C07K 5/04, 7/06, A61K 37/02</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/14115</b> <b>(43) International Publication Date:</b> 22 July 1993 (22.07.93)
<b>(21) International Application Number:</b> PCT/EP92/01060 <b>(22) International Filing Date:</b> 14 May 1992 (14.05.92)  <b>(30) Priority data:</b> 819,893                      16 January 1992 (16.01.92)      US  <b>(71)(72) Applicant and Inventor:</b> PORRO, Massimo [IT/IT]; Via Selvapiana, 97, I-53040 Rapolano Terme-Siena (IT).  <b>(74) Agent:</b> BARZANO & ZANARDO MILANO S.P.A.; Via Borgonuovo 10, I-20121 Milan (IT).  <b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MW, NL, NO, RO, RU, SD, SE, UA, US, European pa- tent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).		<b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> SYNTHETIC PEPTIDES FOR DETOXIFICATION OF BACTERIAL ENDOTOXINS AND TREATMENT OF SEPTIC SHOCK  <b>(57) Abstract</b>  The present invention provides novel peptides of the formula: $R_1-(A-B-C)_n-R$ , wherein $R_1$ and $R$ are independently H or an amino acid residue or a fatty acid residue; A is an amino acid residue selected from the group consisting of Lys, Arg, and His; B is an amino acid selected from the group consisting of Phe, Tyr and Trp; C is an amino acid selected from the group consisting of Leu, Ile and Val; n is an integer of 1-100. The peptides are used <i>inter alia</i> for the prevention and/or treatment of septic shock, for the detection of endotoxin and the preparation of antigenic complexes of Lipid A.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LI	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

## SYNTHETIC PEPTIDES FOR DETOXIFICATION OF BACTERIAL ENDOTOXINS AND TREATMENT OF SEPTIC SHOCK

Shock, which is induced by endotoxin, is known as septic shock (SS). This condition is a life-threatening situation which occurs following infections by Gram-negative bacteria as complication of surgery, prolonged hospitalization, accidents and other traumatic events. It is today well recognized that the agent responsible for this disease is the bacterial endotoxin, a glycolipid antigen present only on the surface of Gram-negative bacteria. This glycolipid is also known as lipo-poly saccharide (LPS) or lipo-oligosaccharide (LOS) depending from the size of the carbohydrate chain which is covalently bound to the fatty-acid-rich moiety called Lipid A (LipA). Only Lipid A is responsible of the major toxic effects shown by endotoxin (LPS). Once endotoxin is released in the blood-stream by bacteria, specialized cells of the immune system like macrophages and monocytes are activated by the endotoxin and several immune mediators are released (Cytokines such as Interleukin-1 and Interleukin-6; ~~IL~~ Tumor necrosis factor;  $\gamma$ - Interferon). Furthermore, endotoxin also activates the complement cascade which results in cell lysis with the consequent release of proteolytic enzymes promoting the release of vasoactive effectors from platelets (e.g.: bradykinine and histamine). The final result is death of the patient in 40-60% of the cases within 48-72 hours. So far, there has been no specific cure or therapy available although bolus injections of adrenal corticosteroids such as methylprednisolone are used.

Polymyxin "B" is known as a molecule that binds and detoxifies bacterial endotoxins and can prevent septic shock when given therapeutically in animal models. However, Polymyxin "B" is a toxic product in vitro and in vivo and this fact limits its potential as a therapeutic agent for the treatment of

septic shock.

Septic shock can be caused by infection with any bacteria that cause the release of LPS. These bacteria include Pseudomonas aeroginosa, Escherichia coli, Salmonella typhi, Neisseria meningitidis, Neisseria gonorrhoeae, Bordetella pertussis, Klebsiella pneumoniae and the like.

The reasons leading to the reported toxicity of Polymyxin B are not completely understood but they are most likely related to the peculiarity of its amino acid composition, specifically for the content of L $\alpha$ - $\gamma$ -, diamino butyric acid (DAB) (49.1% w/w of the structure) which is an analog of the aa Lysine (reported in literature as able to substitute Lysine in the protein synthesis) and for the presence of D-Phenylalanine an isomer of the naturally occurring L-Phenylalanine. Other possible reasons, still related to the aa composition, could be related to the high stability of Polymyxin "B" to proteolytic enzymes as well as to the possible binding to cell receptors structurally comparable to the Lipid A moiety of LPS (the gangliosides of the nervous tissues are glycolipids with N,O - acyl (C<sub>14</sub>-C<sub>18</sub>) chains closely related to the N,O - acyl chains present in the Lipid A structure).

The applicants have discovered new conformational peptides that are structurally different from Polymyxin (in virtue of their amino acid composition) but are capable of binding to the same binding site within Lipid A of endotoxins (LOS and LPS) that Polymyxin "B" will also bind. The relative binding efficiency of the new peptides is comparable to the affinity constant value of Polymyxin "B". The complex formed when Lipid A or LPS are reacted with the peptides of the invention is non-toxic and the natural antigenicity of Lipid A and LPS is maintained.

As a consequence of this high-affinity binding to the Lipid A moiety of endotoxins, most of the synthetic peptide analogs have shown the ability to detoxify endotoxins as evidenced by in vitro as well as in vivo analysis. The in vitro test used, as measure of detoxification, the inhibition of the enzymatic cascade leading to the coagulation of the Lymulus lysate (LAL test) by endotoxin. The LAL test is recognized as the most sensitive and predictive test for the toxic and pyrogenic activity of LPS, since pyrogenicity in vivo is related to the release of the endogenous immune modulators Interleukin-1 (IL-1) and alfa-Tumor necrosis factor ( $\alpha$ -TNF), the mediators responsible for the fatalities associated to septic shock. As an in vivo test confirming detoxification of LPS, was then used the Rabbit pyrogen test performed according to the United States Pharmacopeia XXI.

This discovery thus provides a new class of compounds that may be used in the treatment of septic shock. It is anticipated that the new peptides will not exhibit in humans the toxic effects of Polymyxin "B", in virtue of their completely natural amino acid composition as well as for their limited resistance to proteolytic degradation in human serum.

Accordingly, it is a primary object of the invention to provide novel prophylactic and therapeutic agents which may be used in the treatment of septic shock.

It is also an object of this invention to provide novel peptide compounds which may be used in the treatment of septic shock.

It is also an object of this invention to provide novel pharmaceutical compositions which may be used in the treatment of septic shock.

It is also an object of this invention to provide novel complexes of Lipid-A or LPS and a peptide

which are antigenic and non-toxic.

It is also an object of this invention to provide a method of producing novel non-toxic Lipid A or LPS antigens.

5 Conditions other than septic shock where an endotoxin is produced may also be treated by the peptides of the invention using the same dose of peptides which is used to treat septic shock. These conditions include pertussis bacterial meningitis and  
10 viral HIV-related infections.

These and other objects of the invention will become apparent from a review of the present specification.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 FIG. 1 is a graph that shows the effect of peptides of the present invention on endotoxin.

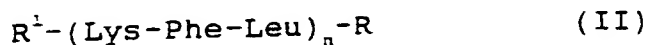
#### DETAILED DESCRIPTION OF THE INVENTION

The invention provides novel monomeric, linear polymeric, cyclic monomeric or cyclic polymeric  
20 peptides of the formula having amphipathic - polycationic characteristics of the formula:



wherein  $R_1$  and  $R$  are independently H or an amino acid residue or a fatty acid residue; A is an amino acid  
25 residue selected from the group consisting of Lys, Arg and His; B is an amino acid selected from the group consisting of Phe, Tyr and Trp; C is an amino acid selected from the group consisting of Leu, Ile and Val; n is an integer of from 1-100, and preferably 1-10.  
30 These peptides are useful in the treatment of septic shock.

A preferred formula according to formula I is formula II:



35 wherein n is an integer of from 1-100 preferably 1-10 and  $R$  and  $R^1$  are H or may be any of the naturally

occurring amino acids or fatty acids with an alkyl chain length encompassing between 1 and 20 (or more) methylene groups; those peptides which have the retro-oriented aa sequences of the described peptides; those peptides which have the enantiomer aa sequences or diastereomer aa sequences of the described peptides; and those peptides which have the aa shifted in place with regard to their original positions which provide a peptide which is useful in the treatment of septic shock.

Examples of peptides of formulas I and II include:

	<u>Group I</u>	<u>Group II</u>	<u>Group III</u>
	(Lys-Phe-Leu)n	(Arg-Phe-Leu)n	(His-Phe-Leu)n
15	(Lys-Phe-Val)n	(Arg-Phe-Val)n	(His-Phe-Val)n
	(Lys-Phe-Ile)n	(Arg-Phe-Ile)n	(His-Phe-Ile)n
	(Lys-Tyr-Leu)n	(Arg-Tyr-Leu)n	(His-Tyr-Leu)n
	(Lys-Tyr-Val)n	(Arg-Tyr-Val)n	(His-Tyr-Val)n
	(Lys-Tyr-Ile)n	(Arg-Tyr-Ile)n	(His-Tyr-Ile)n
20	(Lys-Trp-Leu)n	(Arg-Trp-Leu)n	(His-Trp-Leu)n
	(Lys-Trp-Val)n	(Arg-Trp-Val)n	(His-Trp-Val)n
	(Lys-Trp-Ile)n	(Arg-Trp-Ile)n	(His-Trp-Ile)n

Specific examples of these peptides include:

Cys-Lys-Phe-Leu-Lys-Lys-Cys  
S - - - - - S

Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys  
S - - - - - S

Lys-Phe-Leu-Lys-Lys-Thr

Ile-Lys-Thr-Lys-Lys-Phe-Leu-Lys-Lys-Thr

Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr-Lys  
S - - - - - S

Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr  
S - - - - - S

5 Ile-Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys  
S - - - - - S

Ile-Lys-Phe-Leu-Lys-Phe-Leu-Lys-Phe-Leu-Lys  
Lys-Phe-Leu-Lys-Phe-Leu-Lys

Arg-Tyr-Val-Arg-Tyr-Val-Arg-Tyr-Val

10 The novel peptides are useful for the  
prophylaxis or treatment of septic shock in mammals  
including humans at doses of about 0.1 $\mu$ g-2.0mg/kg of  
body weight or may be used at a level of about 10 $\mu$ g to  
about 0.1mg/kg of body weight and the amount may be  
15 administered in divided doses on daily basis. The  
peptides may be administered prophylactically to  
patients who may be exposed to or have been exposed to  
organisms which may cause septic shock or to detoxify  
bacterial endotoxins by the use of the same dose set  
20 forth above in vivo. In vitro detoxification or  
prevention of endotoxin contamination may be carried  
out at a level of which is effective to achieve the  
desired result. The amount may be based on routine  
experimentation based on the premise about 1 mole of  
25 endotoxin is bound by 1 mole of peptide as shown in  
Table III. The particular dose of a particular peptide  
may be varied within or without the range that is  
specified herein depending on the particular  
application or severity of a disease and the condition  
30 of the host. Those who are skilled in the art may

ascertain the proper dose using standard procedures.

The compounds may be administered intravenously and parenterally using well known pharmaceutical carriers or inert diluents. Oral administration is not preferred because the peptides will tend to be degraded by the enzymes of the alimentary tract. Water or isotonic saline are preferred diluents and a concentration of 0.1 mg per ml may be used. Preferably, the compounds will be stored in a dry form and will be dissolved in the diluent immediately prior to administration.

The novel peptides may be synthesized by classical methods of peptide chemistry using manual or automated techniques as well as by DNA recombinant technology. The synthetic procedure comprises solid phase synthesis by Fmoc chemistry, cleavage (TFA 95%+Et-(SH)<sub>2</sub> 5%), followed by vacuum evaporation. Thereafter, the product is dissolved in 10% acetic acid, extracted with ether, concentrated at 0.1 mg/ml at pH of 6.0-7.5. Stirring under filtered air followed for 1 to 6 hours in case of the Cysteine-containing peptides and finally desalting by reverse phase chromatography is carried out.

Generally, the complexes of Lipid-A and LPS with the peptides of the invention may be made using stoichiometric amounts of Lipid-A or LPS with the peptide. The amounts of complex also able to induce antibody in a host are not critical; about 1 mcg of Lipid-A in the complex with the peptide has been shown to be effective in safely inducing antibodies in a host.

The activity of the peptides has been confirmed by the direct microprecipitin assay with B. pertussis Lipid A, and B. pertussis LPS. In addition, the binding activity for LPS as compared to Polymyxin "B" has been demonstrated on the basis of the ratio of

peptide/LPS and peptide/Lipid A on a w/w basis. The data from the Limulus (LAL) test shows that the novel compounds, when tested at a proper concentration, have equivalent LAL inhibition to Polymyxin "B".

5           The invention also includes the use of the peptide to contact systems containing endotoxin dispersed in a fluid for the purpose of detoxifying the endotoxin. This procedure may be used to detoxify biopharmaceuticals such as vaccines, solutions of  
10       drugs, injectable nutrient solutions, and the like. The invention further comprises the use of the peptides as additives for fluids which will support bacterial growth that will produce endotoxin. The presence of the non-toxic peptide will detoxify any endotoxin which  
15       is subsequently elaborated.

          The peptides of the invention have not been shown to exhibit in vitro the peculiar antibiotic activity of polymyxin B against clinically relevant bacteria such as Vibrio cholerae, Salmonella Typhi and  
20       Haemophilus influenzae at concentrations as high as 1mg/ml. The novel peptides disclosed herein have not shown hemolytic activity on human red blood cells ex vivo at concentrations of as high as 1 mg/ml.

          The peptides have not exhibited acute  
25       toxicity in vivo when injected in Swiss Webster mice at 50 mg/kg after 48 hours observation and beyond. The LD<sub>50</sub> for polymyxin B is 2.5-5 mg/kg for the same species of mice.

          No abnormal toxicity has been shown in mice  
30       or guinea pigs following i.p. injection according to the US CFR Title 21 610.11(b). The test animals were observed for seven days or beyond and did not exhibit any signs of abnormality.

          In addition, the novel compounds have been  
35       shown to be relatively unstable in the presence of proteolytic enzymes such as trypsin while it has been

confirmed that Polymyxin "B" is stable in the presence of trypsin. These results show that the novel compounds are useful for the treatment of septic shock.

#### DESCRIPTION OF THE PREFERRED EMBODIMENTS

5           The following exemplifies the preferred procedure for the synthesis of the compounds of the invention.

          Using the following procedure, peptides have been synthesized using the automatic synthesizer  
10       MILLIGEN Mod. 9050 (MILLIPORE, Burlington, MA) on a solid phase support of polyamide/Kieselguhr resin (2.0g). The amino acids used in the synthesis of the peptide analogs were Fmoc-aa-Opfp derivatives (9-Fluorenylmethyloxycarbonyl-aa-O-pentafluorophenyl  
15       ester) of each amino acid (aa) involved in the considered sequences using 0.8 mmol of each amino acid to sequentially form the peptide.

          Each cycle of synthesis was performed at r.t. (20°C) and involved the following steps of reaction:

#### 20       Step 1 - Deprotection

          The first aa Fmoc-protected at the amino group, was treated with a 20% solution of piperidine for 7 minutes in order to remove the Fmoc-protecting group. Washing with dimethylformamide followed for 12 minutes  
25       to remove all traces of piperidine. Deprotection and washing were run continuously through the column containing the resin by mean of pump at a flow of 5 ml/min.

#### 30       Step 2 - Activation of the Fmoc-aa-Opfp derivative

          The amino and carboxy-protected amino acid due, according to the desired sequence, was activated after its dissolution in 5 ml of dimethylformamide, by catalytic amount of hydroxybenzotriazol (0.5 ml of a 5% w/v solution in dimethylformamide).

#### 35       Step 3 - Acylation

          The activated and protected Fmoc-aa-Opfp derivative was

then recycled for 30 minutes through the column by the pump at 5 ml/min in order to obtain coupling of the introduced aa at the ~~α~~-amino group (previously deprotected as reported in Step 1) of the amino acid preceding the new one in the desired sequence.

Step 4 - Washing

Washing of the matrix in the column followed by dimethylformamide for 2 minutes at 5 ml/min before a new cycle began.

At the completion of the synthesis, the peptide on the resin support was cleaved by 95% Trifluoroacetic acid (TFA) with 5% Ethane dithiol as scavenger, if Cysteine residues were present in the aa sequence, at room temperature for 2 hours. After separation of the cleaved peptide from the resin by filtration, the solution was concentrated by vacuum evaporation to dryness. The collected solid residue was then solubilized in 10% acetic acid at a concentration of 10-20 mg/ml and several extractions by diethyl ether followed (six to eight extractions with half of the volume of the peptide solution) in order to remove the scavenger Ethane dithiol. The peptide solution was then neutralized by 0.1 N ammonium hydroxide and adjusted to the concentration of roughly 0.1 mg/ml. The solution was then stirred under air for 1 to 6 hours. in order to obtain the selective oxidation of the two sulphhydryl groups belonging to the Cys residues of the sequence. In this way, only monomeric oxidized peptides were obtained with no traces of polymeric material. The solution of oxidized peptide was then desalted by reverse-phase chromatography on SEP-PAK C-18 cartridges (MILLIPORE) and finally freeze-dried. The products were analyzed by high-performance liquid chromatography (HPLC) analysis as well as by chemical analysis of the synthetic structures.

-11-

Fast Atom Bombardment Mass Spectrometry was used to confirm the calculated mass of the peptides.

The following peptides were prepared using the procedure which has been set forth above:

- 5 I Cys-Lys-Phe-Leu-Lys-Lys-Cys  
S - - - - - S
- II Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys  
S - - - - - S
- III Lys-Phe-Leu-Lys-Lys-Thr
- 10 IV Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr-Lys  
S - - - - - S
- V Cys-Lys-Lys-Leu-Phe-Lys-Cys-Lys-Thr  
S - - - - - S
- VI Ile-Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys  
15 S - - - - - S
- VII Ile-Lys-Thr-Lys-Lys-Phe-Leu-Lys-Lys-Thr
- VIII Ile-Lys-Phe-Leu-Lys-Phe-Leu-Lys-Phe-Leu-Lys
- IX Lys-Phe-Leu-Lys-Phe-Leu-Lys
- X Arg-Tyr-Val-Arg-Tyr-Val-Arg-Tyr-Val

20 The amino acid composition of each peptide was determined by PICO-TAG after acid hydrolysis by 6N hydrochloric acid for 1-12 hours at 150°C and was found to be as follows:

Table I

25	PEPTIDE	AMINO ACID	AMINO ACID COMPOSITION <sup>1</sup> (moles aa/mol peptide)	
			EXPECTED	FOUND
30	I	Cys	2.00	2.13
		Leu	1.00	1.06
		Lys	3.00	2.90
		Phe	1.00	1.01
35	II	Cys	2.00	2.16
		Leu	1.00	0.99
		Lys	5.00	4.95
		Phe	1.00	0.96
		Thr	1.00	1.03

-12-

	III	Leu	1.00	0.98
		Lys	3.00	2.99
		Phe	1.00	1.01
		Thr	1.00	1.05
5	IV	Cys	2.00	2.15
		Leu	1.00	0.94
		Lys	5.00	4.97
		Phe	1.00	0.93
		Thr	1.00	1.10
10	V	Cys	-	1.85
		Leu	-	0.94
		Lys	-	4.04
		Phe	-	0.98
		Thr	-	1.06
15	VI	Cys	2.00	2.14
		Ile	1.00	0.98
		Leu	1.00	0.99
		Lys	5.00	4.98
		Phe	1.00	0.94
20		Thr	1.00	1.00
	VII	Ile	1.00	0.98
		Leu	1.00	1.00
		Lys	5.00	4.99
		Phe	1.00	0.98
25		Thr	2.00	2.00
	VIII	Ile	1.00	0.98
		Leu	3.00	2.98
		Lys	4.00	3.92
		Phe	3.00	3.02

calculated by the ratio (on molar basis) between the amount of each peptide and the amount of Lipid A present in the structure of LPS used in the experiments:

5

Table IIISTOICHIOMETRY OF THE COMPLEXES FORMED BETWEEN LPS<sub>bp</sub>

AND

SYNTHETIC PEPTIDE ANALOGS OF POLYMYXIN "B"

	Amount of peptide <sup>**</sup> in the complex (nmoles)	Ratio peptide/LipA (mol/mol)
10		
	Polymyxin "B"	2.69
	Peptide II	3.39
	Peptide IV	3.55
15	Peptide VI	3.12
	Peptide VII	3.00
	Peptide VIII	3.86

To further characterize the binding activity of the synthetic peptides for Lipid A of endotoxin, experiments of direct competition with Polymyxin "B" have been set-up in order to evaluate the Affinity constant value of Polymyxin "B" for the toxic moiety of endotoxin and ultimately to calculate the Selectivity of the synthetic peptide analogs (ratio on molar basis, between the affinity constant value of a given peptide and that of Polymyxin "B" for Lipid A). Table IV shows the relative values of Affinity and those of

<sup>\*</sup>Complexes formed between 10 µg of B. Pertussis LPS (equivalent to 4.50 µg of Lipid A or 2.64 nmoles) and 10 µg of peptide (twice the amount corresponding to the saturation point found for Polymyxin "B" in the analysis of AFFINITY)

<sup>\*\*</sup>Values represent the average of two separate experiments of amino acid analysis after acid hydrolysis of the recovered complexes.

-13-

5	IX	Leu	2.00	1.90
		Lys	3.00	3.10
		Phe	2.00	1.90
	X''	Arg	3.00	3.00
		Tyr	3.00	2.95
		Val	3.00	2.90

10 All peptides of the above reported formulas were compared with Polymyxin "B" in a direct microprecipitin assay for Lipid A and LPS of B. Pertussis (5  $\mu$ g each) in order to detect their precipitating (binding) activity:

		<u>Table II</u>		
		<u><math>\mu</math>g</u>	<u>nmol</u>	<u>Complex</u>
	<u>ppt</u>			
15	Polymyxin "B"	7.3	6.1	+ + +
	Peptide I	5.3	6.1	+ + -
	Peptide II	7.5	6.1	+ + +
	Peptide III	4.7	6.1	+ - -
	Peptide IV	7.5	6.1	+ + +
20	Peptide V	7.5	6.1	+ + +
	Peptide VI	8.2	6.1	+ + +
	Peptide VII	7.5	6.1	+ + +
	Peptide VIII	8.7	6.1	+ + +

25 Quantitation of the amount of precipitated peptides present in the complexes with LPS of B. pertussis has been done by amino acid analysis after acid hydrolysis (by 6 M HCl) of the complexes recovered by centrifugation at 3,000 rpm x 15 minutes. In Table III, the stoichiometry of some complexes is reported as

30 "Peptide X was cleaved from the resin overnight at r.t. by 95% trichloroacetic acid containing 5% phenol as a scavenger.

Selectivity for the investigated peptides:

Table IV

CHARACTERISTICS OF THE COMPLEXES FORMED BETWEEN LPS<sub>bp</sub>  
AND

5 SYNTHETIC PEPTIDE ANALOGS OF POLYMYXIN "B"

	Peptide	AFFINITY (K <sub>a</sub> )	SELECTIVITY	AMOUNT OF
		(L/Moles)	(K <sub>a</sub> <sub>ANA</sub> /K <sub>a</sub> <sub>PCP</sub> )	ppt <sup>*</sup>
	Polymyxin "B"	1.15 x 10 <sup>7</sup>	1.0	+ + +
	Peptide I	< 1.15 x 10 <sup>5</sup>	< 0.01	+ + -
10	Peptide II	0.56 x 10 <sup>7</sup>	0.49	+ + +
	Peptide VI	0.29 x 10 <sup>7</sup>	0.25	+ + +
	Peptide IV	0.49 x 10 <sup>7</sup>	0.43	+ + +
	Peptide VII	0.19 x 10 <sup>7</sup>	0.17	+ + +
	Peptide VIII	1.29 x 10 <sup>7</sup>	1.12	+ + +
15	Peptide IX	0.1 x 10 <sup>7</sup>	0.10	+ + +
	Peptide X	0.27 x 10 <sup>7</sup>	0.24	+ + +

The results obtained by the Limulus (LAL) test, shown in Table V, support the data obtained by measuring the Affinity of the peptides of the invention for the Lipid A moiety of LPS in that they were substantially equivalent to Polymyxin "B" in the inhibition of LPS activity on Limulus. The only peptide that showed a lower activity in the LAL inhibition was Peptide I which gave the lowest affinity constant value among the peptides reported in the present invention. Peptide I was, in fact, the one presenting the non complete structure needed for the mimick of Polymyxin "B" as the synthetic peptide analogs II, IV, VI and VII have clearly shown in the previous Table IV. It is important to note that the LAL test is accepted by the most important institutions

<sup>\*</sup>Detected as amount of precipitate obtained by microprecipitation in capillary tubes and by immunodiffusion in agarose.

-16-

in the Public Health field (World Health Organization, United States Food and Drug Administration, etc.) as a predictive test for absence of pyrogenicity in injectable material and it can be used to replace the in vivo test of pyrogenicity in rabbits.

Table V

INHIBITION OF LPS-INDUCED GELATION IN LAL TEST BY SYNTHETIC PEPTIDES MIMICKING THE STRUCTURE OF POLYMYXIN "B"

	LPS/Pept	TEST**
LPS (0.1 µg LPS)		POSITIVE
Polymyxin "B" (0.1 µg + LPS (0.1 µg))	1	NEGATIVE
Peptide I (0.1 µg) + LPS (0.1 µg)	1	POSITIVE
Peptide I (1.0 µg) + LPS (0.1 µg)	10	NEGATIVE
Peptide I (10.0 µg) + LPS (0.1 µg)	100	NEGATIVE
Peptide II (0.1 µg) + LPS (0.1 µg)	1	NEGATIVE
Peptide III (100 µg) + LPS (0.1 µg)	1000	POSITIVE
Peptide IV (0.1 µg) + LPS (0.1 µg)	1	NEGATIVE
Peptide VI (0.1 µg) + LPS (0.1 µg)	2	NEGATIVE
Peptide VII (0.1 µg) + LPS (0.1 µg)	2	NEGATIVE
Peptide IX	100	NEGATIVE
Peptide X	20	NEGATIVE

	(w/w)	
LPS (0.1 µg LPS)		POSITIVE
Polymyxin "B" (0.1 µg + LPS (0.1 µg))	1	NEGATIVE
Peptide I (0.1 µg) + LPS (0.1 µg)	1	POSITIVE
Peptide I (1.0 µg) + LPS (0.1 µg)	10	NEGATIVE
Peptide I (10.0 µg) + LPS (0.1 µg)	100	NEGATIVE
Peptide II (0.1 µg) + LPS (0.1 µg)	1	NEGATIVE
Peptide III (100 µg) + LPS (0.1 µg)	1000	POSITIVE
Peptide IV (0.1 µg) + LPS (0.1 µg)	1	NEGATIVE
Peptide VI (0.1 µg) + LPS (0.1 µg)	2	NEGATIVE
Peptide VII (0.1 µg) + LPS (0.1 µg)	2	NEGATIVE
Peptide IX	100	NEGATIVE
Peptide X	20	NEGATIVE

The results indicate that in order to mimic the structure of Polymyxin "B" for efficiently binding and detoxifying LPS, a synthetic peptide needs to have almost the complete aa sequence of Polymyxin "B" (Peptides II, IV, VI and VII contain ten and eleven aa residues versus ten aa residues of Polymyxin "B") with analogous (but not identical) chemical features. In contrast Peptide III, which contains only six aa residues (the linear sequence of the peptide-cycle in Polymyxin "B") is not able to efficiently bind and

\*The test had a sensitivity of 0.125 Endotoxin Units/ml equivalent in our case (LPS of B. Pertussis) to 0.4 ng/ml of LPS. The complexes were allowed to form at 37°C for 30 minutes before to be processed for analysis after dilution 1/100 with saline.

\*\*Values are representative of a minimum of three different analysis.

detoxify LPS. The minimal structure able to detoxify LPS appears to be Peptide I (corresponding to the peptide-cycle of Polymyxin "B") which, however, does not show an Affinity value comparable to the other peptide analogs showing a longer aa sequence.

The effects of trypsin present in human serum on Polymyxin "B" and the peptides of the invention was determined by combining 10  $\mu$ l of human serum with 20  $\mu$ g of the given peptide in 10  $\mu$ l volume and holding the mixture at a temperature of 37°C for different intervals of time. At various times, an aliquot of the mixture was processed by HPLC analysis in order to detect the residual amount of the investigated peptide. In Table VI the half-lives time of each peptide investigated are shown as compared to the half-life time of Polymyxin "B".

TABLE VI

STABILITY OF SYNTHETIC PEPTIDE ANALOGS OF POLYMYXIN "B"  
TOWARDS PROTEOLYSIS BY TRYPSIN IN HUMAN SERUM

	Half-Life Time	AMOUNT RECOVERED (%)
<u>Peptide</u>	<u>(t/2) (min)</u>	<u>after 180 mins</u>
<u>(%)</u>		
Polymyxin "B"	>> 180	100
Peptide I	> 180	70
Peptide II	50	10
Peptide VI	1,080 (18 hours)	76
Peptide IV	18	0
Peptide V	240	55
Peptide VII	50	28
Peptide VIII	7	0
Peptide IX	10	0
Peptide X	35	0

Tryptic hydrolysis of Peptide VI generates Peptide II

Tryptic hydrolysis of Peptide IV generates Peptide V

As already mentioned in the background of the invention, the pyrogenic activity of LPS in vivo is due to the release from macrophages and monocytes of the cytokines Interleukin-1 (IL-1) and  $\propto$ -Tumor Necrosis Factor ( $\propto$ -TNF) the leading molecules responsible for the fatal effects of septic shock.

In order to verify "in vivo" the detoxifying activity of the peptides, we have injected five groups of three rabbits each with the complexes formed by two representative synthetic peptide analogs with LPS. The pyrogenicity test has been executed according to the United States Pharmacopeia (Vol. XXI)/The National formulary (Vol. XVI), Combined Edition, January 1, 1985. As a negative control in the test, the complex formed by Polymyxin "B" and LPS was injected. As a positive control free LPS was injected. The results are reported in the Fig. 1. As one can see, LPS has shown its peculiar pyrogenic activity starting the first hour from the injection and the temperature continued to increase until the third hour of observation as required by the test. The peculiar behavior of a febrile pattern induced by LPS, involves two waves of temperature increase (biphasic behavior): The first temperature increase (first wave) it is shown within two hours from the injection of LPS and it is due to the immediate impact of the antigen on the host's immune system. The second and more consistent temperature increase (second wave) appears in the third hour from the injection of LPS and it is mediated by the endogenous pyrogens IL-1 and  $\propto$ -TNF released from the immune competent cells stimulated by LPS. The two complexes formed with LPS by the Peptide I and Peptide II as well as by Polymyxin "B" did not show either of the two waves of temperature increase, demonstrating that the two immune mediators IL-1 and  $\propto$ -TNF were not released in vivo upon injection of (complexed)

pyrogenic doses of LPS. The results are shown in FIG. 1.

The following experiments compared the antibiotic activity of Polymyxin "B" with various peptides of the invention.

The tests were performed on BHI plates with liquid cultures of the test organism to give a lawn. Each peptide was diluted in water and placed on sterile Wathmam 3M disks on the surface of the plate. The plates were dried and incubated at 37°C. The zone of inhibition was measured after 18 hours:

Compound	Concentration mg/ml	Zone (mm) of inhibition		
		<u>S. typhi</u>	<u>H. influenzae</u>	<u>V. cholerae</u>
Polymyxin "B"	1.0	4	6	5
	0.2	2	3	2.5
	0.04	1	0	2
	0.008	0	0	1
Peptide I	1.0	0	0	0
	0.2	0	0	0
	0.04	0	0	0
	0.008	0	0	0
Peptide II	1.0	0	0	0
	0.2	0	0	0
	0.04	0	0	0
	0.008	0	0	0
Peptide VI	1.0	0	0	0
	0.2	0	0	0
	0.04	0	0	0
	0.008	0	0	0

The effect of the peptides of the invention on LPS-induced polyclonal B-cell activation was demonstrated by culturing spleen cells from unimmunized healthy SJL/J mice with 50 µg/ml of LPS and Polymyxin "B" or the peptides of the invention at the indicated concentrations. Cells were cultured in RPMI medium containing 1.0% normal mouse serum at 37°C for 3 days. Cultures were pulsed with 1.0µi/well of 3H-thymidine for 16 hours and harvested for counting on an LS betaplate counter. The results were as follows:

-20-

	Units ( $\mu\text{g/ml}$ )	$^3\text{H}$ -thymidine incorporation (cpm)		
		PmB	Peptide I	Peptide II
	none	22,737	22,737	22,737
	100	4,128	3,287	2,266
5	50	2,831	2,775	2,355
	25	3,559	2,582	2,445
	12.5	2,366	2,385	2,350

cpm measured with non stimulated cultures = 2,449.

The binding efficiency of Peptide II to the endotoxin which is elaborated by clinically important gram negative bacteria was demonstrated by the LAL test. The results are shown in Table VII:

	SOURCE OF ENDOTOXIN	EU/ml IN REACTION	PEPTIDE/LPS (w/w)	TEST	EFFICIENCY** OF BINDING (%)
15	B. Pertussis	4	1	Negative	> 98
	E. Coli 055:B5	4	1	Negative	> 98
	P. Aeruginosa	4	1	Negative	> 98
	S. Typhosa	4	1	Negative	> 98
	K. Pneumoniae	4	1	Negative	> 98
20	S. Minnesota	4	1	Negative	> 98
	S. Marcescens	4	1	Negative	> 98
	S. Flexneri	4	1	Negative	> 98
	E. Coli 0111:B4	4	1	Negative	> 98
	V. Cholerae	4	1	Negative	> 98

\* Average of three replicative analysis

\*\* Efficiency of binding > 98% corresponds to < 0.08 Eu/ml of free endotoxin (NEGATIVE LAL TEST).

Efficiency of binding of only 97% corresponds to 0.12 EU/ml of free endotoxin (POSITIVE LAL TEST).

Peptide VI of the invention was labeled with Biotin which acts as a sensitive marker to provide a bi-specific molecule able to selectively react with Lipid A of bacterial endotoxins through Peptide VI ( $K_a = 0.3 \times 10^7$ ) and with the high affinity natural protein Avidin through the labeling molecule Biotin ( $K_a = 10^{15}$ ). The combination of the two selective and high affinity reactions, allows detection of Lipid A of endotoxins at very low levels (picomolar level or  $10^{-12}$  Moles/liter).

The reaction of Biotin-Avidin is used as an example for detecting the reaction between Lipid A/LPS and one of the peptides of the invention.

5           Peptide VI was conjugated to N-hydroxy-succinimidyl Biotin (1:1 mol/mol) in 0.1M sodium acetate solution at pH=6.0. The reaction was kept at 37°C for 1 hour. In these conditions only the -amino group of the amino terminal aa (Ile) reacts so that the  
10           resulting peptide is monosubstituted and does not lose affinity for Lipid A. The labeled peptide was purified by reverse-phase liquid chromatography (HPLC) and chemically analyzed for aa composition and free amino groups. Analysis confirmed that biotinylation of the peptide occurred at the ratio 1:1 mol/mol.

15           Affinity for Lipid A/LPS and half-life time in human serum or human whole blood of the labeled Peptide VI (when tested according to the methods described herein were found not significantly different from the values reported in the same application ( $K_a =$   
20            $0.3 \times 10^7$  Moles/litre and  $t/2 = 20$  hours, respectively).

            Affinity of the peptide bound-Biotin for Avidin, was found not significantly different from the one detected for free Biotin. At equivalent concentrations (1 nmol/ml) free and peptide-bound  
25           Biotin competed similarly for Avidin, as estimated by inhibition of the reaction between peroxidase-labeled Biotin and Avidin in a solid-phase DOT-BLOT assay on nitrocellulose.

            By virtue of the found stoichiometry of the  
30           complex peptide/Lipid A (1:1 mol/mol) and that one known for the complex Biotin/Avidin (4:1 mol/mol), it becomes possible to estimate an unknown amount of endotoxin in a given sample, by titration of the amount of the labeled peptide which is bound to endotoxin and  
35           which is revealed by the reaction between the labeling agent (i.e. Biotin) and its specific reagent (i.e.

enzyme-labeled Avidin).

The results demonstrate the preparation of a novel high sensitive and selective reagent able to reveal even traces of endotoxin in fluids (i.e. serum, blood and aqueous solutions).

5 Lipid A and LPS derived from B. pertussis have been detoxified with the stoichiometric amount of Peptide II and injected in mice respectively at the dose of 1 and 2  $\mu$ g with and without 1 mg/dose of the adjuvant aluminum hydroxide. The immunization schedule included three doses given subcutaneously, three weeks apart. At the end of the immunization period, sera of the 10 mice/group were pooled and analyzed for the presence of antibodies (IgG and IgM isotypes) specific for the Lipid A moiety of endotoxin, at each stage of the immunization period (week 0, 3, 6 and 8).

10 Titers were analyzed for specificity and quantitative amount of antibodies by solid phase assay (DOT-BLOT on nitrocellulose). Nitrocellulose sheets were coated with Lipid A or LPS at 10 or 20  $\mu$ g/ml in PBS pH=7.2 for 7 hours at room temperature. After washing the nitrocellulose with PBS containing 3% BSA w/v, the sera pool of mice was incubated at various dilutions with the Lipid-A-coated nitrocellulose, overnight at room temperature. Then, the Peroxidase-labeled anti-IgG or anti-IgM antibody was added for 2 hours at room temperature, followed by repetitive washing and by the substrate 4-chloronaphthol at 0.3% w/v. The enzymatic reaction was developed for 0.5 - 1 hour at room temperature in the dark.

25 Results of the anti-IgG and anti-IgM titers in the sera pool of mice, are reported in Tables VIII and IX. They show that when Lipid A as well as LPS are injected in a mammalian host in the form of complexes, after detoxification by the peptides of the invention, their natural antigenic repertoire is still intact and

a specific serologic response is generated by the host's immune system. No antibodies were induced that were specific for the peptide present in the complex injected. Animals did not show any sign of hemorrhagic lesions or skin necrosis at the sites of injection after each dose of the complexes.

Thus, the peptides of the invention provide a novel method for the modification of a toxic antigen like Lipid A or LPS which may be used in a mammalian host in the form of safe, non-toxic complexes expressing the natural and specific antigenic repertoire of the bacterial endotoxin to induce immunity to the mammalian host.

Antibodies may be recovered from the antiserum using conventional procedures such as ammonium sulfate or alcohol precipitation and affinity-chromatography, in order to use the isolated Lipid A/LPS-specific antibodies for diagnostic use in fluids as well as for treatment of septic shock in a host.

#### TABLE VIII

##### Anti-Lipid A IgG Response

(sera pool of mice treated with Lipid A  
or LPS detoxified with Peptide II)

Week	<u>Dilution<sup>-1</sup></u>	<u>Dilution<sup>-1</sup></u>
	(with Al(OH) <sub>3</sub> )	(without Al(OH) <sub>3</sub> )
0	0	0
3	50	50
6	100	50
8	200	100

TABLE IXAnti-Lipid A IgM Response

(sera pool of mice treated with Lipid A or LPS  
detoxified with Peptide II)

5	<u>Dilution<sup>-1</sup></u>		<u>Dilution<sup>-1</sup></u>
	<u>Week</u>	<u>(with Al(OH)<sub>3</sub>)</u>	<u>(without Al(OH)<sub>3</sub>)</u>
	0	0	0
	3	50	25
	6	200	50
10	8	100	50

Prevention of endotoxin-induced death in mice, has been achieved by intravenous injection of the peptides of the invention. For this experiment, a strain of mice highly sensitive to the lethal activity of bacterial endotoxin has been used. Mice sensitized with Actinomycin D (Strain CD1) show a high sensitivity to extremely low doses of endotoxin. A dose as low as 1 µg of endotoxin per mouse (about 40 µg/kg of body weight) is able to completely kill a population of mice within 24-48 hours.

Groups of 20 mice CD1 have been treated intravenously with the peptides of the invention, with a single dose of 0.1 mg peptide, solubilized in sterile saline, per mouse. Thirty minutes later, mice were challenged by intraperitoneal injection of 1 µg of endotoxin purified from E. Coli strain 055-B5. Surviving mice were recorded every 24 hours during a seven days-period of observation. Parallel experiments were performed using comparable doses of Polymyxin B (PmB) and Chlorpromazine (CPZ, an anti-histaminic drug recently shown to be highly effective in preventing lethality in this strain of mice by challenge of endotoxin), as positive controls. Negative controls received an intravenous injection of saline.

Table X shows the results obtained: the

survival rate of the mice treated by the peptides of the invention followed a behavior predictable from the affinity constant value of the peptides for Lipid A (see Table IV).

TABLE X

SURVIVAL RATE IN CD1 MICE SENSITIZED WITH ACTINOMYCIN D

		24	48	72	96	120	144	168 hs.	Significance
10	NaCl	5 (25%)	3 (15%)	1 (5%)	1 (5%)	1 (5%)	1 (5%)	1 (5%)	
	Peptide I	8 (40%)	4 (20%)	3 (15%)	3 (15%)	3 (15%)	3 (15%)	3 (15%)	p < 0.02
15	Peptide II	13 (65%)	8 (40%)	8 (40%)	8 (40%)	8 (40%)	8 (40%)	8 (40%)	p < 0.001
	Peptide VI	5 (25%)	5 (25%)	5 (25%)	5 (25%)	5 (25%)	5 (25%)	5 (25%)	p < 0.01
	PmB	10 (50%)	8 (40%)	6 (30%)	6 (30%)	6 (30%)	6 (30%)	6 (30%)	p < 0.001
20	CPZ	10 (50%)	10 (50%)	10 (50%)	10 (50%)	10 (50%)	10 (50%)	10 (50%)	p < 0.001

There were 20 mice per group. Mice surviving at each of the seven 24 hours observation periods are listed. The % survival appears in parenthesis. P expresses the level of statistical significance calculated by "t-Test" for each molecule compared to the treatment with saline, considering the total survival rate in each group.

Peptide II shows a higher efficacy in comparison to PmB (p < 0.05).

Peptide II shows the same efficacy of CPZ (p < 0.2).

Another experiment, performed in mice (Strain Balb/c) naturally resistant to high doses of endotoxin (up to 0.5 mg/mouse), gave further evidence of the safety and efficacy of the peptides of the invention with respect to a comparable treatment performed with Polymyxin B.

Groups of 20 mice Balb/c have been treated

intravenously with the peptides of the invention at the dose of 1 mg/mouse or with 0.1 mg/mouse of Polymyxin B (the highest dose of this drug tolerated in the mouse, when injected alone). Thirty minutes later, mice were challenged by intraperitoneal injection of 1 mg endotoxin from E.C strain 055-B5. Surviving mice were recorded every 24 hours during a seven days-period of observation. Negative controls received an intravenous injection of saline.

Table XI shows the results obtained: treatment of the animals by the peptides of the invention, resulted safe and efficacious. By contrast, treatment with Polymyxin B resulted efficacious only within three days following the endotoxin challenge, since immediately thereafter the toxicity of Polymyxin B (PmB) played a synergistic role with endotoxin and all mice died.

TABLE XI  
SURVIVAL RATE IN BALB/c MICE

	24	48	72	96	120	144	168 hs.	Significance
NaCl	12 (60%)	10 (50%)	8 (40%)	8 (40%)	8 (40%)	8 (40%)	8 (40%)	
Peptide I	18 (90%)	12 (60%)	10 (50%)	10 (50%)	10 (50%)	10 (50%)	10 (50%)	p < 0.01
Peptide II	20 (100%)	12 (60%)	12 (60%)	12 (60%)	12 (60%)	12 (60%)	12 (60%)	p < 0.001
PmB	18 (90%)	14 (70%)	12 (60%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	n.s.

There were 20 mice per group. Mice surviving at each of the seven 24 hours observation periods are listed. The % survival appears in parenthesis. P expresses the level of statistical significance calculated by "t-Test" for each molecule compared to the treatment with saline, considering the total survival rate in each group.

Peptide I and Peptide II show safety and

efficacy in comparison to PmB ( $p < 0.001$ ).

Comparative Example

5 In further support of the features described for the peptide of Claim I, and required for the binding activity to Lipid A, a peptide of the formula:

10           Glu-Tyr-Val-Glu-Tyr-Val-Glu-Tyr-Val  
analog of the Peptide X but showing poly-anionicity rather than poly-cationicity (Arg residues replaced by Glutamic acid residues) was synthesized and showed  
15 neither binding activity for Lipid A/LPS nor inhibition of the toxic activity of LPS in the LAL assay.

15 The peptides of the invention may be used in conjunction with Polymyxin-B at level which is in a stoichiometric excess of the Polymyxin-B calculated on the basis of the selectivity shown in Table IV in order to reduce the toxicity of Polymyxin B.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Porro, Massimo
- (ii) TITLE OF INVENTION: Synthetic Peptides for Detoxification  
of Bacterial Endotoxins and for the  
Prevention and Treatment of Septic  
Shock
- (iii) NUMBER OF SEQUENCES: 10
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Hedman, Gibson, Costigan & Hoare
  - (B) STREET: 1185 Avenue of the Americas
  - (C) CITY: New York
  - (D) STATE: New York
  - (E) COUNTRY: USA
  - (F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 Mb storage
  - (B) COMPUTER: IBM PS/2
  - (C) OPERATING SYSTEM: DOS
  - (D) SOFTWARE: Word Perfect 5.1
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Costigan, James V.
  - (B) REGISTRATION NUMBER: 25,669
  - (C) REFERENCE/DOCKET NUMBER: 576-002
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (212) 302-8989
  - (B) TELEFAX: (212) 302-8998

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:

-29-

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Lys Phe Leu Lys Lys Cys  
1 5

(3) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Lys Thr Lys Cys Lys Phe Leu Lys Lys Cys  
1 5 10

(4) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Lys Phe Leu Lys Lys Thr  
1 5

(5) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Cys Lys Lys Leu Phe Lys Cys Lys Thr Lys  
1 5 10

(6) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids

-30-

(B) TYPE: amino acid  
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Lys Lys Leu Phe Lys Cys Lys Thr  
1 5

(7) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids  
(B) TYPE: amino acid  
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Ile Lys Thr Lys Cys Lys Phe Leu Lys Lys Cys  
1 5 10

(8) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 10 amino acids  
(B) TYPE: amino acid  
(C) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Ile Lys Thr Lys Lys Phe Leu Lys Lys Thr  
1 5 10

(9) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11 amino acids  
(B) TYPE: amino acid  
(C) TOPOLOGY: circular

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ile Lys Phe Leu Lys Phe Leu Lys Phe Leu Lys  
1 5 10

(10) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7 amino acids

-31-

(B) TYPE: amino acid  
(C) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Lys Phe Leu Lys Phe Leu Lys  
1 5

(11) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids  
(B) TYPE: amino acids  
(C) TOPOLOGY: linear

(ii) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Arg Tyr Val Arg Tyr Val Arg Tyr Val  
1 5

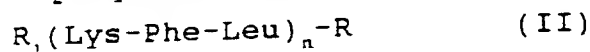
CLAIMS

1. A monomeric, linear polymeric, cyclic monomeric or cyclic polymeric peptide of the formula:



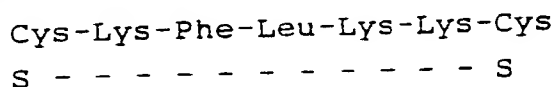
wherein  $R_1$  and  $R$  are independently H or an amino acid residue or a fatty acid residue; A is an amino acid residue selected from the group consisting of Lys, Arg and His; B is an amino acid selected from the group consisting of Phe, Tyr and Trp; C is an amino acid selected from the group consisting of Leu, Ile and Val;  $n$  is an integer of from 1-100.

2. A monomeric, linear polymeric, cyclic monomeric or cyclic polymeric peptide of the formula:

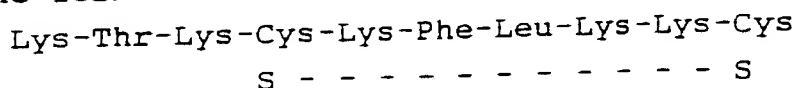


wherein  $n$  is a integer of from 1-10 and  $R$  and  $R_1$  are H or an amino acid residue or a fatty acid residue.

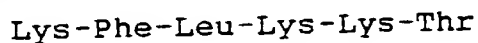
3. A peptide according to claim 1 which is of the formula:



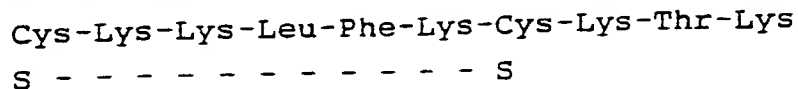
4. A peptide according to claim 1 which is of the formula:



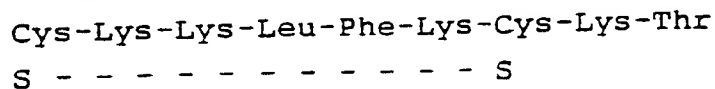
5. A peptide according to claim 1 which is of the formula:



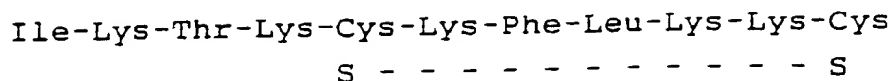
6. A peptide according to claim 1 which is of the formula:



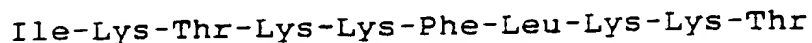
7. A peptide according to claim 1 which is of the formula:



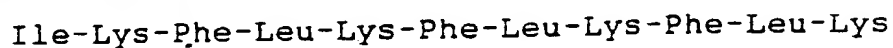
8. A peptide according to claim 1 which is of the formula:



9. A peptide according to claim 1 which is of the formula:



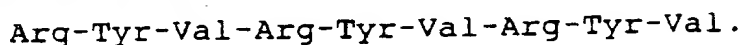
10. A peptide according to claim 1 which is of the formula:



11. A peptide according to claim 1 which is of the formula:



12. A peptide according to claim 1 which is of the formula:



13. A pharmaceutical composition which comprises a peptide of claim 1 and a pharmaceutical carrier.

14. A pharmaceutical composition which comprises a peptide of claim 2 and a pharmaceutical carrier.

15. A pharmaceutical composition which comprises a peptide of claim 3 and a pharmaceutical carrier.

16. A pharmaceutical composition which comprises a peptide of claim 4 and a pharmaceutical carrier.

17. A pharmaceutical composition which comprises a peptide of claim 5 and a pharmaceutical carrier.

18. A pharmaceutical composition which comprises a peptide of claim 6 and a pharmaceutical carrier.

19. A pharmaceutical composition which comprises a peptide of claim 7 and a pharmaceutical carrier.

5 20. A pharmaceutical composition which comprises a peptide of claim 8 and a pharmaceutical carrier.

10 21. A pharmaceutical composition which comprises a peptide of claim 9 and a pharmaceutical carrier.

22. A pharmaceutical composition which comprises a peptide of claim 10 and a pharmaceutical carrier.

15 23. A pharmaceutical composition which comprises a peptide of claim 11 and a pharmaceutical carrier.

24. A pharmaceutical composition which comprises a peptide of claim 12 and a pharmaceutical carrier.

20 25. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 1.

25 26. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 2.

27. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 3.

30 28. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 4.

29. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 5.

35 30. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 6.

31. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 7.

5 32. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 8.

10 33. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 9.

34. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 10.

15 35. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 11.

36. A method of treating septic shock which comprises administering to a host an effective amount of a peptide of claim 12.

20 37. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 1 to a susceptible host.

25 38. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 2 to a susceptible host.

39. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 3 to a susceptible host.

30 40. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 4 to a susceptible host.

41. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 5 to a susceptible host.

35 42. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 6 to a susceptible host.

43. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 7 to a susceptible host.

5 44. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 8 to a susceptible host.

10 45. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 9 to a susceptible host.

46. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 10 to a susceptible host.

15 47. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 11 to a susceptible host.

48. A method of preventing septic shock which comprises administering an effective amount of a peptide of claim 12 to a susceptible host.

20 49. A method of reducing the toxicity of Polymyxin B which comprises administering an effective amount of a peptide of claim 1 in combination with Polymyxin B.

25 50. A method for removal of endotoxin from human and animal blood or sera which comprises contacting said blood or sera with a peptide of claim 1.

30 51. A method for the control of the release of the cytokines induced by endotoxin which comprises administering an effective amount of a peptide of claim 1 to a host.

52. Peptide sequences which are the retro-oriented aa sequences of claim 1.

35 53. Peptide sequences which are the enantiomer aa sequences (all-D aa in the sequence) of the peptides of claim 1.

54. Peptide sequences which are the diastereomer aa sequences of the peptides of claim 1 ( -D and -L aa in the same sequence).

5 55. Peptide sequences in which the amino acids are inverted with respect to their original position in the sequence of the peptides of claim 1.

10 56. A method for the detoxification of bacterial endotoxins which comprises treating the affected host with an effective amount of the peptide of claim 1.

15 57. A method for the use of the peptides of claim 1 as diagnostic probes for detection and quantitation of endotoxin in sera or blood of mammals as well as in solutions which comprise labeling the peptide with a sensitive marker useful for the specific detection of endotoxin; contacting said sera or blood with the labeled peptide and determining the presence of endotoxin.

20 58. A method for the preparation of a non-toxic, antigenic complex of Lipid A or LPS which comprises contacting Lipid-A or LPS with a peptide of Claim 1 and thereafter recovering the antigenic complex.

25 59. A method for preparing antibodies to Lipid A or LPS which comprises the steps of (a) contacting Lipid-A or LPS with a peptide of Claim 1 to form a complex; (b) administering an effective amount of said complex to an host; and (c) recovering antibodies from the serum of said host.

30 60. A method of inducing antibodies to Lipid-A or LPS in a host which comprises the steps of (a) contacting Lipid-A or LPS with a peptide of Claim 1 to form a complex; and (b) administering a effective amount of said complex to said host.

35

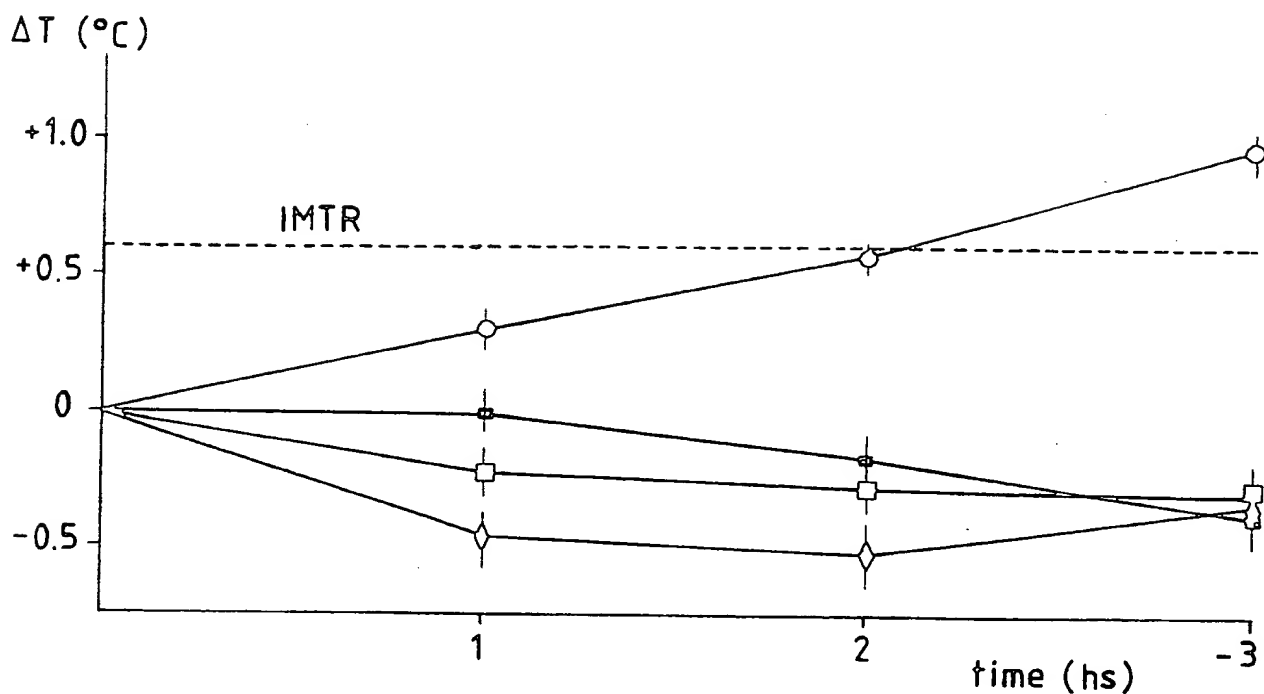
61. A method for the detoxification of a bacterial endotoxin which comprises contacting the bacterial endotoxin or a fluid containing the endotoxin with an effective amount of the peptide of Claim 1.

5

62. A method for preventing contamination of a product with endotoxin, said method comprising adding to a product an amount of a peptide of Claim 1 which is sufficient to neutralize any endotoxin which is subsequently elaborated by bacterial growth.

10

1/1



The value 0 corresponds to the mean of the control temperature ( $T=39.54\pm0.05^{\circ}\text{C}$ ), for the twelve rabbits tested.

- positive control: endotoxin 30 ng/Kg body weight
- negative control: endotoxin 30 ng/Kg body weight complexed with 60 ng Polymyxin "B"
- endotoxin 30 ng/Kg body weight complexed with 60 ng Peptide II.
- ◇ endotoxin 30 ng/Kg Body weight complexed with 300 ng Peptide I.

IMTR: Individual Maximal Temperature Rise allowed by the U.S. Pharmacopeia (vol. XXI), The National Formulary (vol. XVI), Combined Edition, January, 1985.

# INTERNATIONAL SEARCH REPORT

International Application No.

PCT/EP 92/01060

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int.Cl. 5 C07K5/04;                      C07K7/06;                      A61K37/02		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	WO,A,9 117 763 (THE UNITED STATES OF AMERICA; THE SECRETARY, U.S. DEPARTMENT OF COMMERCE) 28 November 1991 * Page 2, line 24 - page 3, line 24 * ---	1-2
X	EP,A,0 304 279 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 22 February 1989 * See page 5, column 2, line 46 * --- <div style="text-align: center;">-/-</div>	1-2
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search <div style="text-align: center;">26 AUGUST 1992</div>	Date of Mailing of this International Search Report <div style="text-align: center;">09.09.92      01.09.92</div>	
International Searching Authority <div style="text-align: center;">EUROPEAN PATENT OFFICE</div>	Signature of Authorized Officer <div style="text-align: center;">KORSNER S.E. </div>	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category <sup>a</sup>	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>CHEMICAL ABSTRACTS, vol. 77, 1972, Columbus, Ohio, US; abstract no. 20028, PAULAY ET AL: 'Antibiotic hexapeptide amides and hydrazides' page 545 ; column 2 ; see abstract &amp; HU,A,3 841 (GYOGYSZERKUTATO INTERZET) 28 March 1972</p> <p>---</p>	54

**Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Although claims 25-49, 51, 56, 59-60 are directed to a method of treatment of the human body, the search has been carried out and based on the alleged effects of the compounds.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO. EP 9201060  
SA 59952

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on  
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 26/08/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9117763	28-11-91	AU-A- 7956791	10-12-91
EP-A-0304279	22-02-89	JP-A- 1131124	24-05-89
HU-A-3841		None	

**THIS PAGE BLANK (USPTO)**